

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
28 October 2004 (28.10.2004)

PCT

(10) International Publication Number
WO 2004/092414 A1

(51) International Patent Classification⁷: **C12Q 1/68**

(21) International Application Number:
PCT/IB2004/001462

(22) International Filing Date: 19 April 2004 (19.04.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
03290958.2 17 April 2003 (17.04.2003) EP

(71) Applicants (for all designated States except US):
**CENTRE NATIONAL DE LA RECHERCHE
SCIENTIFIQUE (CNRS) [FR/FR];** 3, Rue Michel
Ange, F-75016 Paris (FR). **UNIVERSITÉ CLAUDE
BERNARD LYON 1 [FR/FR];** 43, bvd du 11 Novembre
1918, F-69100 Villeurbanne (FR).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **BEZIN, Laurent,**
Georges, Bernard [FR/FR]; 122, rue Saint-Georges,
F-69005 Lyon (FR). **MORALES, Anne, Catherine**
[FR/FR]; 12 E Impasse Lafontaine, F-69500 Bron (FR).

(74) Agents: **MARTIN, Jean-Jacques et al.;** Cabinet Regim-
beau, 20, rue de Chazelles, F-75847 Paris Cedex 17 (FR).

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD,
MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG,
PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM,
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM,
ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), Euro-
pean (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR,
GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:

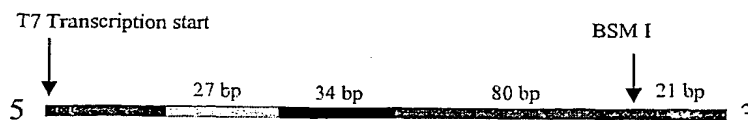
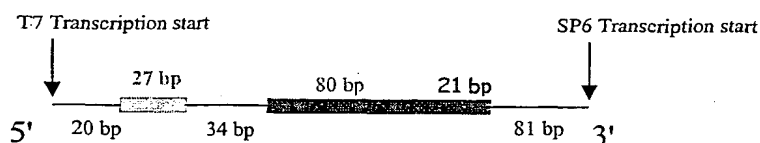
— of inventorship (Rule 4.17(iv)) for US only

Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: METHOD OF CALIBRATION OF REVERSE TRANSCRIPTION USING A SYNTHETIC MESSENGER RNA (SM-
RNA) AS AN INTERNAL CONTROL



(57) Abstract: The invention pertains to the field of reverse transcription reactions and relates to a new method of calibration with a synthetic messenger RNA (SmRNA). It is aimed at a synthetic messenger RNA (SmRNA) designed for normalization of reverse transcription reaction of mRNAs of a biological sample, wherein said SmRNA does not compete with target mRNAs amplification and does not interfere with the reverse transcription of endogenous mRNAs of said sample.

**Method of calibration of reverse transcription
using a synthetic messenger RNA (SmRNA)**

- 5 The invention pertains to the field of reverse transcription reactions and relates to a new method of calibration with a synthetic messenger RNA (SmRNA).

Quantitative RT-PCR assays involve simultaneously amplifying control molecules and samples containing a target mRNA. Known amounts of a control molecule are
10 thermally cycled with tubes, wells or slides, containing the unknown amount of target. In addition to the pair of PCR primers for the target, a pair of PCR primers is required for the control molecule. Following amplification, the amounts of amplified products are compared. However, inherent variation in amplification efficiency among primers results in poor quality of results.

15

More recently, this method has been improved. Kits comprising fluorescent dyes and quenchers are provided by Applied Biosystems and are available under the trademark TaqManTM. Improvements allowed the development of real-time
quantitative PCR.

20

These methods have been reviewed for example in Heid et al, Real-time quantitative PCR. Genome Research. 1996; 6: 986-994, and in Gibson et al, A novel method for real time quantitative RT-PCR Genome research. 1996; 6 : 995-1001. Despite the use of different sets of tubes containing triplicate two-fold
25 dilutions of a control molecule and a fixed amount of unknown target, variation in the quantification between samples still occurs.

We found in connection with the invention that such drawback is due to the nature of the control molecule used. In Heid et al, the internal standard used for
30 normalization is an endogenous gene such as β -actin. Endogenous genes are not appropriate since they may vary from one sample to the other and can interfere with amplification of related gene sequences. In Gibson et al, it is proposed to use a random sequence selected from the target sequence or a modified sequence deriving

from the target sequence. In this case, variation between sample may also occur and it would require the design of one control molecule for each target sequence, which makes it impossible when analyzing the expression of large number of genes.

- In Wang et al, Quantification of mRNA by the polymerase chain reaction, Proc. Natl. Acad. Sci. USA, 1989, 86 : 9717-9721, it is suggested to use a standard whose sequence comprise complementary sequence of the target gene so that one pair of primer is sufficient to amplify both products. Again, such approach is irrelevant with the analysis of a large number of genes. A similar method is described in WO 00/20629.

10

In these competitive quantitative RT-PCR methods, PCR primers sequences for the standard are strictly identical to the PCR primers of the target mRNA.

There are mainly two alternatives regarding the standard:

- 15 - Its sequence is homologous to the mRNA internal sequence and will be referred as "competitive homologous external standard". It is also commonly called "internal standard". Such internal standard enters in competition with the target mRNA in course of the PCR reaction (see for example Gibson et al, Genome Research, vol. 6 No 10, Oct. 1996 p. 995-1001; WO 91/02817; WO 01/16367; Siebert et al, Nature, 20 vol. 359, Oct. 1992, p. 557-558; Zamorano et al, Neuroendocrinology, vol. 63, 1996, pages 397-407; Jensen et al, Journal of Immunological Methods, vol. 215, No 1-2, June 1998, p. 45-58; Sheflin et al, Endocrinology, Vol. 132, No 6, 1993, p. 2319-2324.
- 25 - Its sequence is homologous to the target mRNA only or mainly for the segment corresponding to the primers. In this case, the standard is called "competitive heterologous external standard". Still, when such standard is added to the reaction mixture, it competes with the amplification of the target mRNA; WO 91/02817.
- 30 In all the above cases, a standard sequence homologous the target mRNA sequence or an standard heterologous sequence with a similar size and G+C content compared to the target is used to achieve a same reverse transcription efficacy between the standard and the target. But, the analysis requires a large series of

reactions with different ratios between the competitive standards and the targets. This is not suitable with high through-put quantification of different transcripts of one sample; Bustin, Journal of Molecular Endocrinology (2000) 25 :169-193 ; p. 173 lignes 44-51.

5

Furthermore, homologous standard are usually shorter than the target mRNA. Considering that the reverse transcription efficacy seems to be dependent more likely to the length rather than to the composition of the RNA (Figure 17), we do not believe that a short homologous standard RNA can be used to determine with
10 precision the reverse transcription efficacy of its target mRNA.

Because quantitative PCR amplifications (real-time PCR) is now widely used for the quantification of the relative alteration in mRNA expression, controlling the RT reaction efficiency using real-time PCR has become an important factor before the
15 further use of the RT products (microarrays, DD-RTPCR ...).

We provide a solution to address the above mentioned problems based on the use of a synthetic messenger RNA (SmRNA) designed for normalization of reverse transcription reaction of mRNAs of a biological sample, which SmRNA does not
20 interfere with the reverse transcription of endogenous mRNA of said sample. Therefore, we provide a new method which is the opposite of the above mentioned prior art competitive methods. Indeed, the method of the invention is based on a non-competitive approach. The goal is not to assess the absolute quantity of a mRNA in a sample. In other words, the invention do not seek to design a standard
25 for a specific target mRNA. Rather, the purpose of the invention is to determine the reproducibility of the reverse transcription of any mRNA using a non-competitive standard. Normalization of the reverse transcription between samples is achieved by calculating the efficacy of the reverse transcription of the SmRNA.

The standard of the invention is a non-competitive heterologous external standard
30 referred as "SmRNA" and is particularly advantageous for the analysis of different genes from different samples using non-competitive real-time quantitative RT-PCR.

Description

Therefore, in a first aspect, the invention relates to a method of quantifying and normalizing products of reverse transcription reaction of mRNA extracted from a biological sample, wherein said method comprises the steps consisting of adding a synthetic messenger RNA (SmRNA) which does not compete with the reverse transcription of target mRNAs of said sample to a reaction mixture comprising mRNA extracted from said sample.

More particularly, the method comprises the steps of :

- 10 a) adding a synthetic messenger RNA (SmRNA) which does not compete with the reverse transcription of target mRNAs of said sample and which do not interfere with the reverse transcription of endogenous mRNA of said sample to a reaction mixture comprising mRNA extracted from said sample;
- b) determining the reverse transcription efficacy ρ for the smRNA and,
- 15 c) adjusting the level of target mRNA transcripts by multiplication with ρ .

Preferably, said SmRNA does not interfere with the reverse transcription of any mRNA of said sample. This is to say that SmRNA displays less than 95%, 97% or 99% or preferably less than 99,50 % identity with the biological sequence of the sample, such as a human sequence, but more generally such as a mammalian sequence, a plant, a bacteria and fungi sequence.

In this method, the reverse transcription efficacy ρ for the smRNA is calculated and is used as a parameter for normalizing reverse transcription of mRNA of different samples, wherein the number of endogenous mRNA transcripts is multiplied by ρ . For example, the addition of 4000 to 65 000 000 copies of SmRNA in 500 ng total RNA allowed to calculate that SmRNA reverse transcription efficacy ρ is about 10% (see Figure 14). ρ is determined by linear regression analysis following $y = (1+\rho) x - C$; wherein x is the quantity of SmRNA and y is the quantity of sCDNA (figure 14).

In frame with the invention, "percent identity" means the percentage of identical nucleotide between two sequences when compared according the best alignments, this percentage being statistical and the differences between the two sequences being at random and on the total length of the sequences. Optimal alignments of sequences can be achieved by means of the algorithm of Smith Waterman (1981) [Ad. App. Math. 2 : 482], with the algorithm for local homology described in Neddleman and Wunsch (1970) [J. Mol. Biol. 48 : 443], with the similarity search method described by Lipman (1988) [Proc. Natl. Acad. Sci. USA 85 : 2444], with softwares using GAP, BESTFIT, FASTA and TFASTA algorithms available in Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI and DNASIS, Version 2.5 for Windows; Hitachi Software Engineering Co., Ltd, South San Francisco, CA, by using the standard parameters described in the manufacturer brochure.

Another possibility is to use the BLAST or FASTDB programs available at WWW.ncbi.nlm.nih.gov with the following parameters "Mismatch penalty 1.00; Gap Penalty 1.00; Gap Size Penalty 0.33; joining penalty 30.0. These algorithms are displayed in Current Methods in Sequencing and synthesis Methods and Applications, pages 127-149, 1988, Ala R. Liss, Inc".

Another feature to define SmRNA of the invention is the fact that it does not hybridize to any mRNA of a given sample in high stringent conditions (for example hybridization in a mixture containing 5 x SSPE, 5x Denhart solution, 0.5% SDS (w/v) and 100 µg/ml salmon sperm DNA].

Said SmRNA allows to perform a reverse transcription reaction and to calibrate the quantity of each target mRNA obtained after amplification with the quantity of the cDNA amplified corresponding to said SmRNA without competition with the target mRNAs and preferably without interfering with reverse transcription of any mRNA of the sample. For example, the addition of 50 119 to 501 000 000 copies of SmRNA to 500 ng total ARN does not interfere with reverse transcribed mRNA coding for GAPDH or BDNF (Figure 15). The addition of 50×10^6 copies of SmRNA to 100 ng (1 UA), 250 ng (2.5 UA) or 500 ng (5 UA) total RNA does not

interfere with reverse transcribed mRNA of GAPDH (1 UA ; 2.5 UA or 5 UA), since the slope of correlation line is ~1.0 (Figure 16).

5 In this method, said SmRNA can be added in different reaction mixtures comprising one sample in different dilutions.

Said SmRNA may further comprise a polyA segment. This allows to perform the reverse transcriptase reaction using a polydT primer for both the target mRNAs and the SmRNA.

10

The SmRNA may be about 80 to 150 nucleotide long or longer, preferably about 100 nucleotide long and comprise particular sequences so as to be specifically amplified with a pair of primers designed to avoid primer dimerization. Short SmRNA is preferred because reverse transcription efficacy is greater for short than
15 for longer RNA (Figure 17). And the greater the reverse transcription efficacy is, the more reliable will be both its determination and the normalization of the efficacy between samples.

The method depicted above may consist of quantitative RT-PCR, DD-RT-PCR, eventually perform in connection with macro or micro-arrays, wherein
20 quantification of target mRNAs is performed by means of normalization with said SmRNA. The invention can be practiced on DNA microarrays. In this case, the cDNA corresponding to the SmRNA is amplified and spotted onto said microarrays. This is an important advantage of the said mRNA, since a same mRNA sequence
25 can be used first to help calibrating the RT reaction in microarrays, and second, to calibrate the RT reaction in real-time RT-PCR (which is a commonly used technique employed following microarrays to validate the results obtained). In other words, both techniques (microarrays and real-time RT-PCR) can use a same standard to control the reaction's efficiency.

30

Alternatively, the method of the invention may consist of Northern blotting wherein said SmRNA is pooled with samples. Furthermore, the SmRNA may be used to calibrate the antisense RNA amplification method (Philips and Eberwine,

"Antisense RNA amplification: a linear amplification method for analyzing the mRNA population from single living cells", Methods, Vol. 10 (1996), pp. 283-288) wherein said SmRNA is pooled with reaction mixture.

- 5 In a particular embodiment, the SmRNA consists of a SEQ ID No 1, SEQ ID No 2, or sequences deriving thereof. It is reverse transcribed into "ScDNA" of SEQ ID No 3 or SEQ ID No 4, preferably using a pair of primer consisting of SEQ ID No 5 and SEQ ID No 6. Such primers are optimized so as to avoid dimerization and are specific for the SmRNA sequence. These SmRNA present a unique sequence to our
10 knowledge when performing a sequence search in Genbank for example. The same applies for the SEQ ID No 3 to 6.

In a second embodiment, the invention relates to a synthetic messenger RNA (SmRNA) designed for normalization of reverse transcription reaction of mRNAs
15 of a biological sample, wherein said SmRNA does not compete with target mRNAs and does not interfere with the reverse transcription of endogenous mRNAs of said sample, which SmRNA displays less than 95% or 99% identity with any biological sequence, comprises a poly A segment and is about 80 nucleotide long or longer, preferably about 100 nucleotide long. In other words, the SmRNA referred herein
20 above does not interfere with the reverse transcription of endogenous mRNA of said sample in the sense that it does not hybridize under stringent conditions to any mRNA sequence of the sample.

This SmRNA may further comprise at least two segments which are complementary
25 to at least two primers that are designed to avoid primer dimerization.

One preferred SmRNA has the sequence shown in SEQ ID No 1 or SEQ ID No 2. Another feature of these particular SmRNA is two segments which are complementary to two primers of SEQ ID No 5 and SEQ ID No 6.

- 30 In this regards, the invention is also directed to a primer or probe selected from of SEQ ID No 5 and SEQ ID No 6.

The invention also encompasses a cDNA obtained from a reverse transcription reaction of a SmRNA as defined above. One particular cDNA consists of SEQ ID No 3 or SEQ ID No 4.

- 5 SmRNAs of the invention may also comprise a segment which is homologous to the target mRNA and are devoid of sequences complementary to the primers sequences used to amplify the target mRNA. Sequences downstream and upstream of this SmRNA can be added for amplifying the cDNA corresponding to the SmRNA (ScDNA) (SEQ ID n° 5 and 6 for example). In this regards, the sequence of the
10 SmRNA will be for the most part homologous to the target mRNA sequence, but this SmRNA will not compete during PCR since primers for the ScDNA and target cDNA are different.

- In another embodiment, the invention concerns a vector comprising a sequence
15 encoding the SmRNA of the invention. The term "vector" refers to a DNA molecule originating from a virus, a bacteria, or the cell of a higher organism into which another DNA fragment of appropriate size can be integrated without loss of the vectors capacity for self-replication; a vector introduces foreign DNA into host cells, where it can be reproduced in large quantities. Examples are plasmids,
20 cosmids, and yeast artificial chromosomes; vectors are often recombinant molecules containing DNA sequences from several sources.

- Preferred vectors are plasmids, more specifically plasmids which comprise the construct as shown in figure 1, notably the sequence SED ID No 7 or SEQ ID No 9
25 and 11. The invention also relates to a vector as depicted in figure 2. More particularly, the invention is directed to a vector as mentioned above which further comprise any sequence corresponding to SEQ ID No 9, 10 and 11 preceded by a sequence corresponding to a RNA polymerase promoter, for example RNA polymerase T7 promoter (sequence ID No 8) and referred as to "DNA probe
30 DNAΣ" (see figure 3).

In still another embodiment, the invention is aimed at a kit for quantification of mRNAs of a biological sample comprising a SmRNA, a vector or a DNA probe as defined above. Such kit may further comprise primers of SEQ ID No 5 and 6.

- 5 The invention also relates to the use of a SmRNA, a vector or a DNA probe as defined above for calibrating target mRNAs during quantification in RT-PCR reaction, more particularly in frame with Q-RT-PCR or DD-RT-PCR, which may optionally be practiced with DNA microarrays.
- 10 The invention also relates to the use of a SmRNA, a vector or a DNA probe as defined above for calibrating target mRNAs in Northern blot analysis or for calibrating antisense RNA amplification method.

The invention is further illustrated in the examples below.

15

EXAMPLE 1: Calibration of the reverse transcription using a synthetic messenger RNA (SmRNA) obtained from a synthetic DNA probe (DNA Σ)

1.1 Characteristics of the DNA probe: DNA Σ

20

- Name of the DNA probe : DNA Σ
- Total Length : 182 bp
- Insertion of a restriction site for BSM I allowing the linearization of the vector just after the poly A sequence
- 25 • Presence of the T7 promoter allowing the in vitro transcription of a synthetic messenger RNA (SmRNA)

1.2 Construction of the DNA probe

- 30 - Insertion of a 101 bp fragment (shown below) in the pGEM®-T Easy Vector with a T4 DNA ligase (Promega).

5'TTCTTCGACTCACTGCAGACTACTGATGGAATGACGTAGTACGAATAC
TCGACTGGTCTCAACATGAAAAAAAAAAAAACGCATTCAACCTGTCTG
ACTA 3' (SEQ ID No 11).

- 5 - Linearization of the cloned plasmid with the Aat II and Sph I restriction enzymes (New England BioLabs®Inc).

3- Insertion of a 27 bp fragment (shown below) with a T4 DNA ligase (Promega).

- 10 5'-CGGGACAAGAAGGTGGAAGACGTCATG-3' (SEQ ID No 9).

The resulting plasmid referred as pGEM®-DNAΣ is shown in Figure 2.

1.3 Synthesis of the DNA probe "DNAΣ"

15

PCR amplification of the plasmid pGEM®-DNAΣ using:

- a mix of HotStarTaq DNA polymerase (Qiagen) and ProofStar DNA polymerase (Qiagen).

- Forward primer A containing the T7 promoter:

- 20 5'TAATACGACTCACTATAGGGCGGGACAAGAAGGTGGAAG3' (SEQ ID No 12)

- Reverse primer A:

5'TAGTCAGACAGGTTGAATGCG3' (SEQ ID No 13)

- 25 Sequence of the DNA probe "DNAΣ"

5'TAATACGACTCACTATAGGGCGGGACAAGAAGGTGGAAGACGTCATG
CTCCCGGCCCGCCATGGCGGCCGCGGGAATTTCGATTTCTTCGACTCACTG
CAGACTACTGATGGAATGACGTAGTACGAATACTCGACTGGTCTCAACA
TGAAAAAAAAAAAAACGCATTCAACCTGTCTGACTA3' (SEQ ID No 7).

30

TAATACGACTCACTATAGGG = sequence of the T7 promoter (SEQ ID No 8)
CGGGACAAGAAGGTGGAAGACGTCATG = 27 bp insert (SEQ ID No 9)

CTCCCGGCCGCCATGGCGGCCGCGGGAATTCGAT = 34 bp from pGEM®-T
Easy sequence (SEQ ID No 10)

TTCTTCGACTCACTGCAGACTACTGATGGAATGACGTAGTACGAATACT
5 CGACTGGTCTCAACATGAAAAAAAAAAAAAAAAACGCATTCAACCTGTCTGA
CTA = 101 bp insert (SEQ ID No 11).

Example 2: *In vitro* transcription of the synthetic mRNA using the plasmid pGEM®-DNAΣ

10

- Linearization of the plasmid pGEM®-DNAΣ with the BSM I restriction enzyme
(New England BioLabs®*Inc*); see Figure 1.

- *In vitro* transcription with T7 RNA polymerase using T7-MEGAshortscript™
15 (Ambion ®).

5'GGGCGAAUUGGGCCCGACGUCGGGACAAGAAGGUGGAAGACGUCAU
GCUCCCGGCCGCCAUGGCGGCCGCGGGAUUCGAUUUCUUCGACUCAC
UGCAGACUACUGAUGGAAUGACGUAGUACGAAUACUCGACUGGUCUC
20 AAC AUGAAAAAAAAAAAAAAAAA 3' (SEQ ID No 1)

Synthetic poly A mRNA #1 (161 nt)

Example 3: *In vitro* transcription of the synthetic mRNA using the DNA probe « DNAΣ »

25

- Digestion of the DNA probe « DNAΣ » with the BSM I restriction enzyme (New
England BioLabs®*Inc*); see Figure 1.

30

- *In vitro* transcription with T7 RNA polymerase using T7-MEGAshortscript™
(Ambion ®).

35

5'UAAUACGACUCACUAUAGGGCGGGACAAGAAGGUGGAAGACGUCAU
GCUCCCGGCCGCCAUGGCGGCCGCGGGAAUUCGAUUUCUUCGACUCAC
UGCAGACUACUGAUGGAAUGACGUAGUACGAAUACUCGACUGGUCUC
AACAUGAAAAAAAAAAAAAAAA 3' (SEQ ID No 2).

5

Synthetic poly A mRNA #2 (161 nt)

Example 4: RT-PCR of both synthetic mRNAs #1 and #2

- 10 **4.1** Reverse transcription of synthetic poly A mRNAs #1 (SEQ ID No 1) and #2
(SEQ ID No 2) with oligo dT using M-MLV Reverse Transcriptase RNase H
Minus, point mutant (Promega) leading to :

Synthetic cDNA #1

- 15 5'GGGCGAATTGGGCCCCGACGTCGGGACAAGAAGGTGGAAGACGTCATG
CTCCCGGCCGCCATGGCGGCCGCGGGAAATTCGATTTCTTCGACTCACTG
CAGACTACTGATGGAATGACGTAGTACGAATACTCGACTGGTCTCAACA
TGAAAAAAAAAAAAAAAA 3' (SEQ ID No 3).

20 and

Synthetic cDNA #2

- 5'TAATACGACTCACTATAGGGCGGGACAAGAAGGTGGAAGACGTCATG
CTCCCGGCCGCCATGGCGGCCGCGGGAAATTCGATTTCTTCGACTCACTG
25 CAGACTACTGATGGAATGACGTAGTACGAATACTCGACTGGTCTCAACA
TGAAAAAAAAAAAAAAAA 3' (SEQ ID No 4).

- 4.2** Amplification of synthetic cDNAs #1 and #2 using PCR on the LightCycler®
(Roche) using the QuantiTect™ SYBR® Green PCR kit (Qiagen) or the LC
30 FastStart DNA Master SYBR Green I (Roche).

Primer III forward (19 nt): 5' CGG GAC AAG AAG GTG GAA G 3' (SEQ ID No
5).

Primer III reverse (22 nt): 5' AGT CTG CAG TGA GTC GAA GAA A 3' (SEQ ID No 6)

The amplified fragment from both synthetic cDNA #1 and cDNA #2 (82 pb) with
5 primer pair III produces the following sequence:

5' GGGACAAGAAGGTGGAAGACGTCATGCTCCCGGCCGCCATGGCGGCC
GCGGGAATTCGATTTCTTCGACTCACTGCAGACT 3' (SEQ ID No 14).

10 **Example 5: Calibration of the RT reaction using SmRNAs of the invention in real-time PCR.**

5.1 We tested several primers to optimize the sequence of the SmRNA as depicted above. The goal was to obtain segments within the sequence which are
15 complementary to pairs of primers which will not dimerize during the reaction while being specific to the SmRNA.

Among the sequences tested, we show here below three examples illustrating the importance of the presence of such segments within the SmRNA of the invention.

20 Pair I: primer forward (20 nt) 5' AATTGGGCCCCGACGTCGCAT 3' SEQ ID No 15 and primer reverse (20 nt) 5' CATGTTGAGACCAGTCGAGT 3' SEQ ID No 16

Pair II: primer forward (19 nt) 5' CGGGACAAGAAGGTGGAAG 3' SEQ ID No 17 and primer reverse (20 nt) 5' TCATGTTGAGACCAGTCGAG 3' SEQ ID No 18

25

Pair III: primer forward (19 nt) 5' CGGGACAAGAAGGTGGAAG 3' SEQ ID No 5 and primer reverse (22 nt) 5' AGTCTGCAGTGAGTCGAAGAAA 3' SEQ ID No 6

30 The left and right primers I, II and III were chosen to minimize as much as possible the PCR priming primer-dimers using the "primer 3" software.

Regarding primers I, control experiments without cDNA show the absence of any amplification, confirming the absence of primer dimerization (curve control, Figure 4). However, these primers did not provide satisfying specificity since (in absence of SmRNA during the RT), they recognized cDNAs obtained from hippocampal tissue (curve cDNA, Figure 4).

Based on these results, it was necessary to design different specific primers. Several specific software, including Primer3, did not allow us to design other primers matching the various cautious required such as the absence of self complementarity and melting temperatures. Therefore, we have inserted a 27 bp fragment in the pGEM®-T Easy Vector with a T4 DNA ligase (Promega) in order to design other primers.

The left and right primers II were chosen to minimize as much as possible the PCR priming primer-dimers. Control experiments without cDNA show the absence of any amplification, which confirms the absence of primer dimerization (curve control, Figure 5). However, the profile of the amplification curve of the pGEM®-DNAΣ, that the primer pair has a weak affinity for the sequence. Other specific primers have thus been designed.

In case of the primers III, control experiments, without cDNA containing the sequence to amplify, show the absence of any amplification, which confirms the absence of primer dimerization (curve control, Figure 6). Furthermore, the amplification curve shows that primers III have a high affinity for the sequence of the pGEM®-DNAΣ, which contains the sequence to amplify.

Using such tests, one can routinely obtain variants of the SmRNA of SEQ ID No 1 and corresponding primers optimized for use as mentioned in the present invention.

5.2 Calibration using primers III and the SmRNA of SEQ ID No 1.

30

5.2.1 Calibration of the amplification of the synthetic cDNA using the LightCycler® (Roche) and the QuantiTect™ SYBR® Green PCR kit (Qiagen).

Several dilutions of the purified synthetic cDNA were amplified with specific primer pair III and the crossing point was determined.

We evidence that the starting concentration of synthetic cDNA before amplification
5 is linearly correlated with the value of the crossing point (validation #1, Figure 7).

5.2.2 Increasing amounts of synthetic mRNA #1 were reverse transcribed and amplified with specific primer pair III using QuantiTect™ SYBR® Green PCR kit (Qiagen) on the LightCycler®

10

We evidence that the amplification of the synthetic cDNA, following reverse transcription of the synthetic mRNA, is dependant of the concentration of the synthetic mRNA #1 reverse transcribed (validation #2, Figure 8).

15 5.2.3 Increasing amounts of synthetic mRNA #1 were reverse transcribed together with 500 ng of total hippocampus RNA of either control or pilocarpine-treated rats. Synthetic cDNA were amplified as above.

We evidence that the dilution of the synthetic mRNA #1 either within total tissue
20 RNA from control or pilocarpine-treated rats does not affect the PCR efficiency (1.68) for synthetic mRNA #1 (validation #3, Figure 9).

5.2.4 Calibration of the amplification of the synthetic cDNA using the LightCycler® (Roche) and LC FastStart DNA Master SYBR Green I (Roche).
25 Several dilutions of the purified synthetic cDNA were amplified and the crossing point was determined.

This calibration curve using the LC FastStart DNA Master SYBR Green I (Roche) is very similar to that obtained with the QuantiTect SYBR Green PCR kit (Qiagen)
30 (see validation # 1, Figure 7), demonstrating that the synthetic cDNA can be equally amplified with the LightCycler using both kits (validation #4, Figure 10).

5.2.5 Calibration of the amplification of the synthetic cDNA using the LightCycler® (Roche) and LC FastStart DNA Master SYBR Green I (Roche). Several dilutions of the purified synthetic cDNA were amplified and the crossing point was determined. In this experiment, the serial dilutions have been performed
5 with a constant concentration of cDNAs obtained from reverse transcription of total brain mRNAs, instead of water (validations # 1 and 4, Figures 7 and 10 respectively).

We evidence that the amplification of the synthetic cDNA is independent of the
10 diluent used (water in validation # 4 = Figure 10 versus brain cDNAs in validation # 5 = Figure 11).

5.2.6 Illustration of the variability of the RT efficiency performed in 86 samples of
15 total RNA from mouse olfactory bulb (OB).

500 ng of total RNA from 86 OB have been reverse transcribed together with 80 pg of SmRNA #1. Reverse transcription has been performed in a PCR Express Thermocycler (Hybaid). cDNA corresponding to the SmRNA #1 has then been quantitatively amplified with primer pair "III" using the LightCycler to control
20 whether identical concentration of cDNA from SmRNA was present in each sample after the reverse transcription step.

Conclusion: The figure 12 shows a wide range of cDNA concentration after reverse transcription, indicating that the reverse transcription was not reproducible across
25 the 86 samples.

5.2.7 Demonstration that SmRNA #1 is appropriate, instead of house-keeping gene GAPDH, to quantify specific mRNA following quantitative relative RT-PCR.

30 500 ng of total RNA from rats (control 2 month old; control 10 month old; DSP-4 treated 10 month old) have been reverse transcribed together with 80 pg of SmRNA #1. cDNAs corresponding to BDNF exon V, GAPDH and SmRNA have been

amplified using gene specific primers with the LightCycler. In each sample, BDNF was either corrected using either GAPDH value or SmRNA value.

Note that GAPDH cannot be used to normalize samples, since its expression is not
5 constant among the three groups tested (Figure 13B). However, SmRNA appears to highlight differences that could not been evidenced before normalization of the reverse transcription (Figure 13A vs. Figure 13D). Furthermore, note that normalization with GAPDH induces biased conclusions (Figure 13B vs. Figure 13D).

10

5.2.8 Influence of the SmRNA #1 addition to the RT reaction the relative variation in the expression of specific genes.

Reverse transcription of 500 ng of total hippocampus RNA from either control or
15 pilocarpine-treated rats. Pilocarpine treatment has been used to stimulate BDNF gene expression in the hippocampus. Exon 5 of the BDNF cDNA was amplified with specific primers and LC FastStart DNA Master SYBR Green I (Roche). BDNF mRNA concentration was quantified using PCR on the LightCycler and the standard curve equation: $y = -3.7621 x + 31.648$.

20

BDNF mRNA concentration in:

Control: 31.5 ± 3.7 A.U.

Pilocarpine: 219.9 ± 36.9 A.U.

25 **Pilocarpine = 6.98 X control**

Reverse transcription of 500 ng of total hippocampus RNA from either control or
pilocarpine-treated rats, together with 80 pg of synthetic mRNA #1 added to the RT
reaction mix. Exon 5 of the BDNF cDNA was amplified with specific primers and
30 LC FastStart DNA Master SYBR Green I (Roche). BDNF mRNA concentration was quantified using PCR on the LightCycler and the standard curve equation: $y = -3.7621 x + 31.648$.

Synthetic cDNA was amplified with specific primers and QuantiTect SYBR Green I PCR kit (Qiagen). Synthetic mRNA #1 concentration was quantified using PCR on the LightCycler and the standard curve equation: $y = -4.717x + 33.569$.

5 BDNF mRNA concentration in:

Control: 31.4 ± 1.1 A.U.

Pilocarpine: 245.5 ± 26.2 A.U.

10 Synthetic mRNA #1 concentration in:

Control: 1.627 ± 0.062 A.U.

Pilocarpine: 1.821 ± 0.053 A.U.

15 BDNF mRNA / synthetic mRNA in:

Control: 19.3

Pilocarpine: 134.8

Pilocarpine = 6.98 X control

20

Conclusion:

The addition of the synthetic mRNA to the reverse transcriptase (RT) reaction mix does not interfere with the reverse transcription of endogenous mRNA.

25 The normalization of BDNF cDNA by the synthetic cDNA allows to recover the same induction profile in pilocarpine-treated rats than that observed without the addition of the synthetic mRNA to the RT reaction mix.

Therefore, we conclude that the addition of the SmRNA of the invention into the
30 RT reaction mix allows a reliable normalization of the RT reaction between samples without competition with target mRNAs.

CLAIMS

- 5 1. A method of normalizing products of reverse transcription reaction of mRNA extracted from different biological samples, wherein said method comprises the steps consisting of
- a) adding a synthetic messenger RNA (SmRNA) which does not compete with the reverse transcription of target mRNAs of said sample and which do not interfere
- 10 with the reverse transcription of endogenous mRNA of said sample to a reaction mixture comprising mRNA extracted from said sample;
- b) determining the reverse transcription efficacy ρ for the smRNA and,
- c) adjusting the level of target mRNA transcripts by multiplication with ρ .
- 15 2. The method of claim 1, wherein a SmRNA displaying less than 95%, 97% or 99% or preferably less than 99,50 % identity with any mRNA of said sample, is used to perform a non-competitive reverse transcription reaction and to calibrate the quantity of each target mRNA obtained after amplification with the quantity of the cDNA amplified corresponding to said SmRNA.
- 20 3. The method of claim 1, wherein a SmRNA unable to hybridize under stringent conditions with any mRNA of said sample, is used to perform a non-competitive reverse transcription reaction and to calibrate the quantity of each target mRNA obtained after amplification with the quantity of the cDNA amplified corresponding
- 25 to said SmRNA.
4. The method of claim 1, wherein the SmRNA comprise a segment which is homologous to the target mRNA and is devoid of sequences complementary to the primers sequences used to amplify the target mRNA.
- 30 5. The method according one of claims 1 to 4, wherein said SmRNA is added in different reaction mixtures comprising one sample in different dilutions.

6. The method according to one of claim 1 to 5, wherein said SmRNA further comprises a polyA segment.
7. The method according to one of claims 1 to 6, wherein said SmRNA is about 80
5 nucleotide long or longer, preferably about 100 nucleotide long.
8. The method according to one of claims 1 to 7, wherein said SmRNA is specifically amplified with a pair of primers designed to avoid primer dimerization.
10. 9. A method according to one of claims 1 to 8 consisting of non-competitive quantitative RT-PCR, wherein quantification of target mRNAs is performed by means of normalization with said SmRNA.
10. A method according to one of claims 1 to 4 consisting of non-competitive DD-
15 RT-PCR, wherein quantification of target mRNAs is performed by means of normalization with said SmRNA.
11. A method according to one of claims 9 and 10 which is performed on DNA microarrays.
- 20 12. A method according to claim 11, wherein cDNA (ScDNA) corresponding to the SmRNA is amplified and spotted onto said microarrays.
13. A method according to one of claims 1 to 11 consisting of Northern blotting
25 wherein said SmRNA is pooled with samples.
14. A method according to one of claims 1 to 13, wherein said SmRNA consists of a SEQ ID No 1 or SEQ ID No 2 or sequences deriving thereof.
- 30 15. A method according to claim 14, wherein said SmRNA is reversed transcribed into cDNA of SEQ ID No 3 using a pair of primer consisting of SEQ ID No 4 and SEQ ID No 5.

16. A synthetic messenger RNA (SmRNA) designed for normalization of reverse transcription reaction of mRNAs of a biological sample, wherein said SmRNA does not compete with target mRNAs and does not interfere with the reverse transcription of endogenous mRNA of said sample, which SmRNA displays less than 95% or 99% identity with any biological sequence of said sample or does not hybridize under stringent conditions to any with any biological sequence of said sample, comprises a poly A segment and is about 80 nucleotide long or longer, preferably about 100 nucleotide long.
17. A SmRNA according to claim 16 which further comprise at least two segments which are complementary to at least two primers that are designed to avoid primer dimerization.
18. A SmRNA according to one of claims 16 and 17 consisting of SEQ ID No 1 or SEQ ID No 2.
19. A SmRNA according to claim 17 comprising two segments which are complementary to at least two primers of SEQ ID No 5 and SEQ ID No 6.
20. A primer or probe selected from of SEQ ID No 5 and SEQ ID No 6.
21. A cDNA obtained from a reverse transcription reaction of a SmRNA according to one of claims 16 to 19.
22. A cDNA according to claim 21 which consist of SEQ ID No 3 or SEQ ID No 4.
23. A vector, more particularly a plasmid, comprising a sequence encoding the SmRNA of one of claims 16 to 19.
24. A vector according to claim 23 which further comprise any sequence corresponding to SEQ ID No 9, 10 and 11 preceded by a sequence corresponding to a RNA polymerase promoter, in particular RNA polymerase T7 promoter (sequence ID No 8) and referred as to "DNA probe DNA Σ " (see figure 3).

25. A vector according to claim 23 or 24 which comprises the construct as shown in figure 1, notably the sequence SED ID No 7 or SEQ ID No 11.
- 5 26. A vector according to claim 25 as depicted in figure 2.
27. A kit for quantification of mRNAs of a biological sample comprising a SmRNA according to one of claims 16 to 19, or a vector according to one of claims 19 to 22.
- 10 28. A kit according to claim 27 further comprising primers of SEQ ID No 5 and 6.
29. The use of a SmRNA according to one of claims 16 to 19 or a vector according to one of claims 23 to 26 for calibrating target mRNAs during quantification in RT-PCR reaction.
- 15 30. The use according to claim 29 in Q-RT-PCR or DD-RT-PCR.
31. The use according to claims 29 or 30 in frame with DNA microarrays.
- 20 32. The use of a SmRNA according to one of claims 16 to 19 or a vector according to one of claims 23 to 26 for calibrating target mRNAs in Northern blot analysis and for calibrating antisense RNA amplification method.

1 / 7

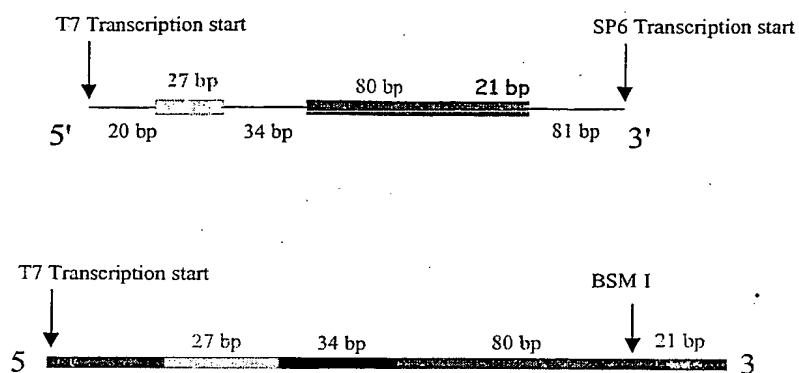


FIGURE 1

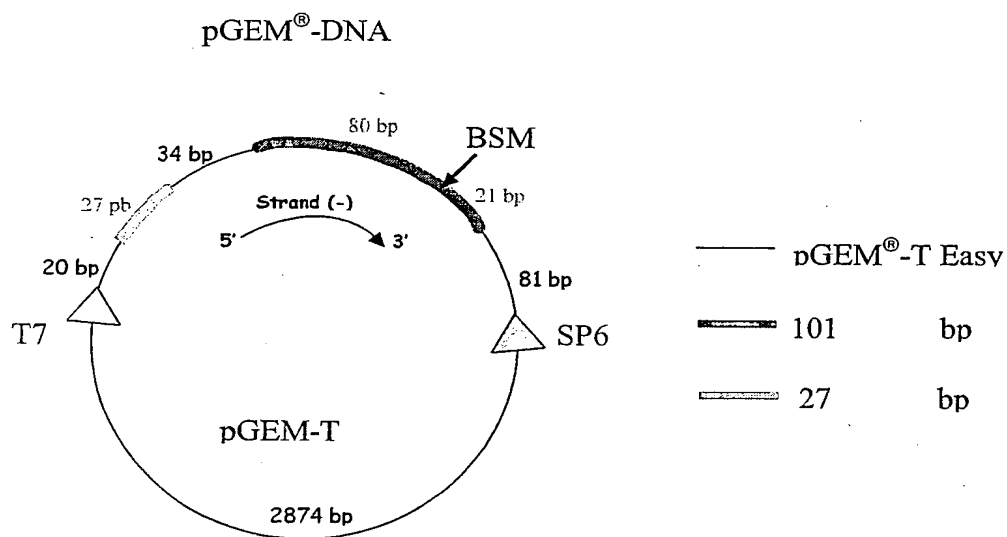


FIGURE 2

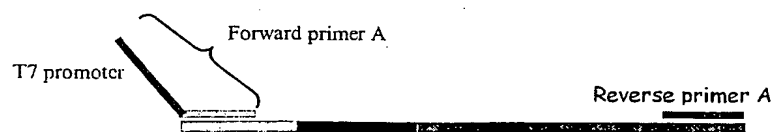


FIGURE 3

2 / 7

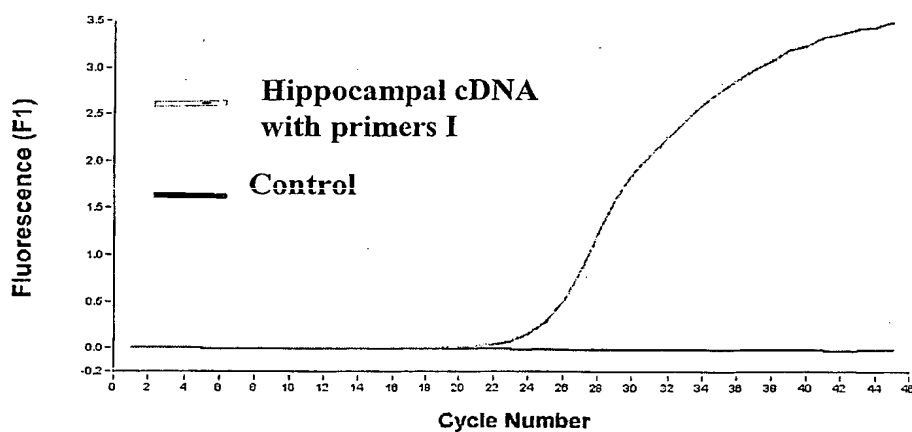


FIGURE 4

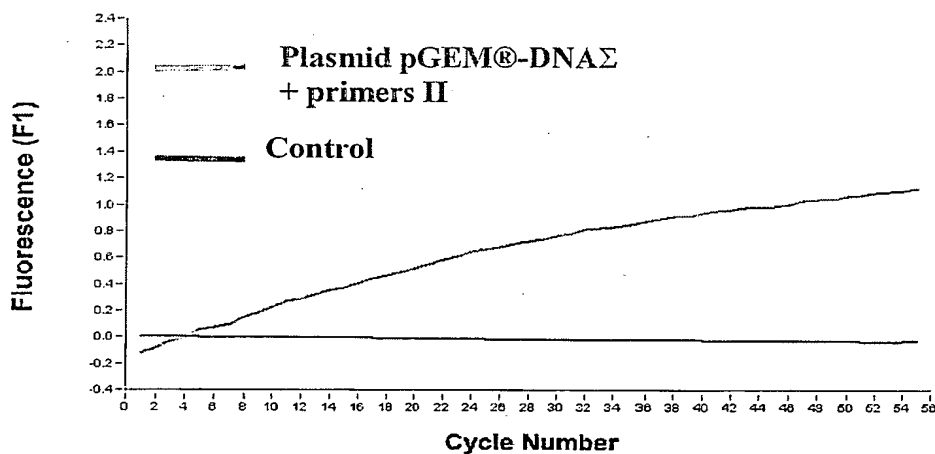


FIGURE 5

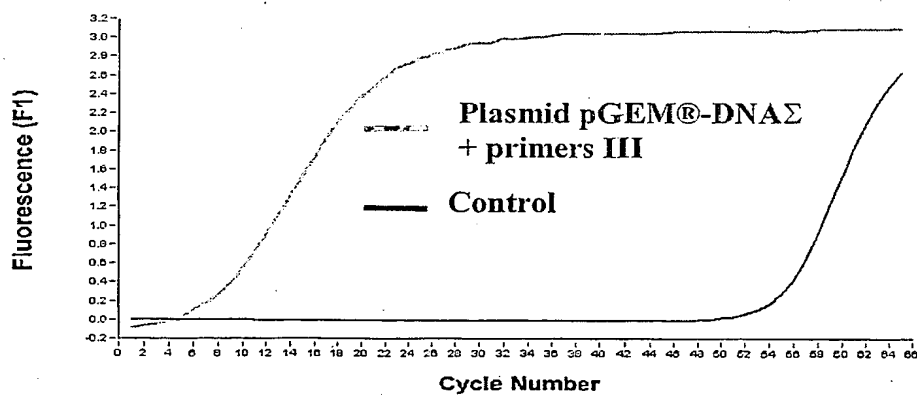


FIGURE 6

3 / 7

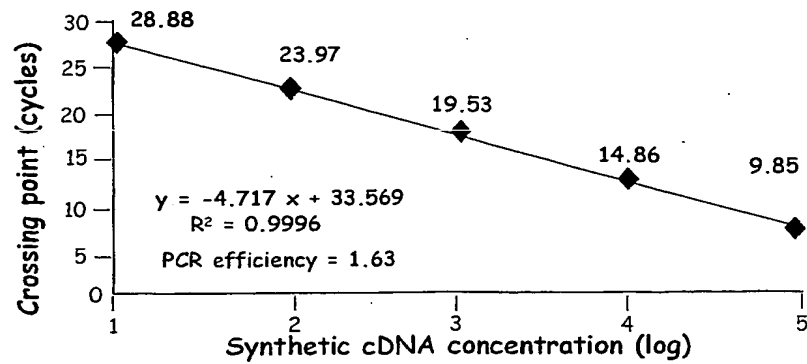


FIGURE 7

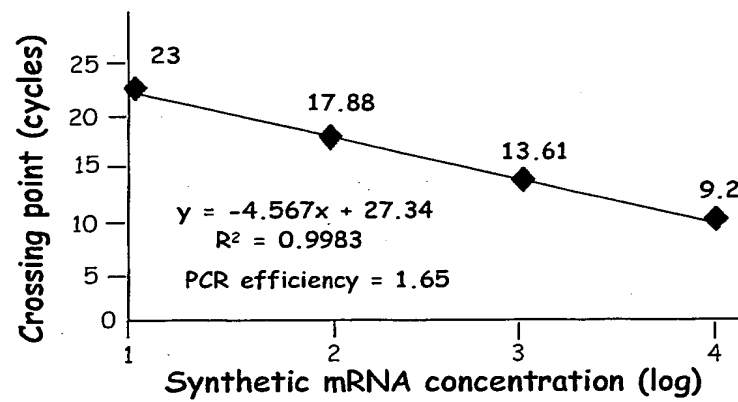


FIGURE 8

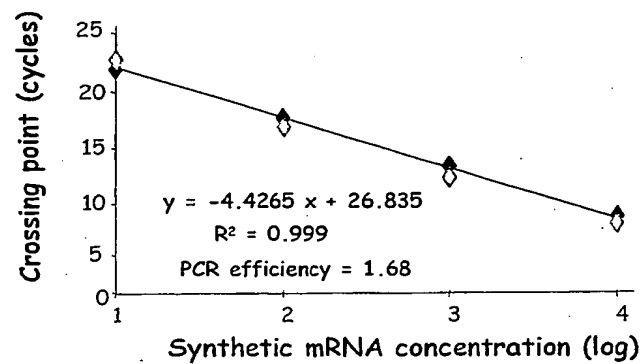


FIGURE 9

4 / 7

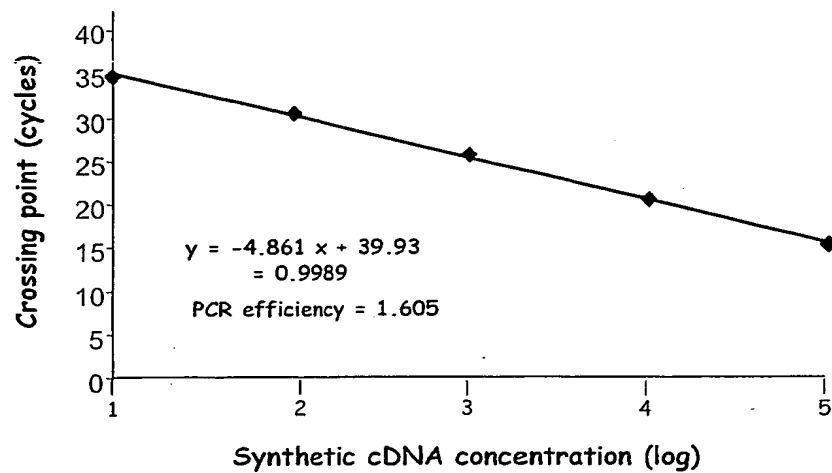


FIGURE 10

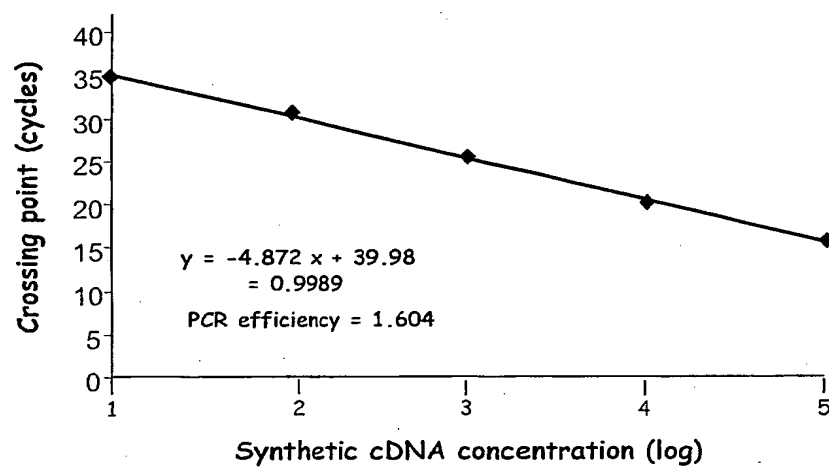


FIGURE 11

5/7

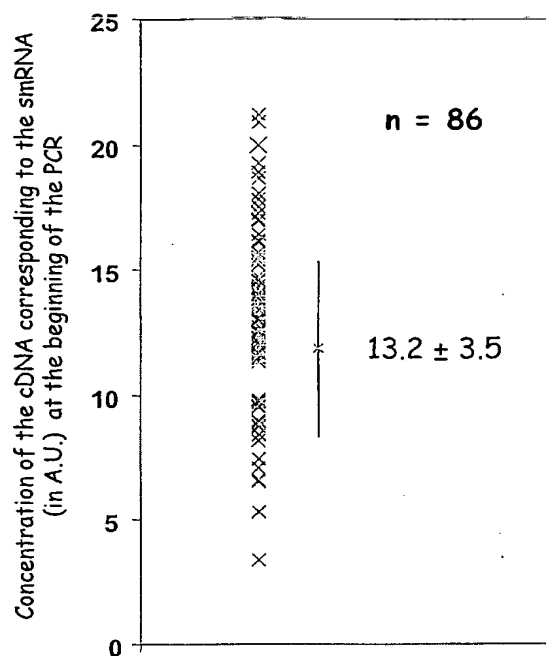


FIGURE 12

FIGURE 13A

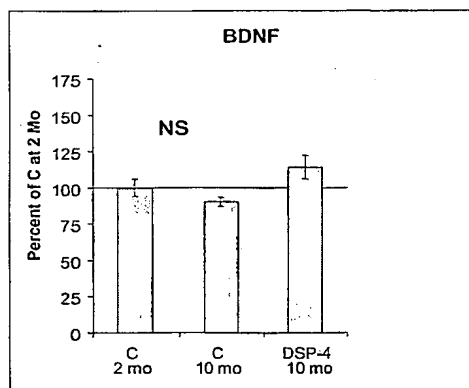


FIGURE 13B

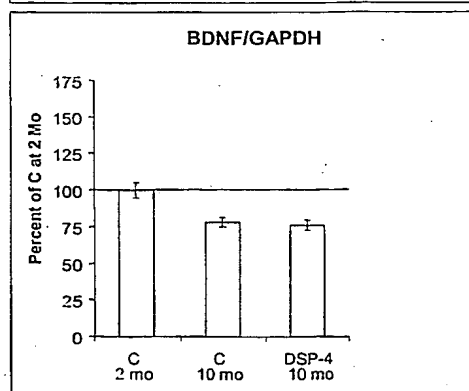
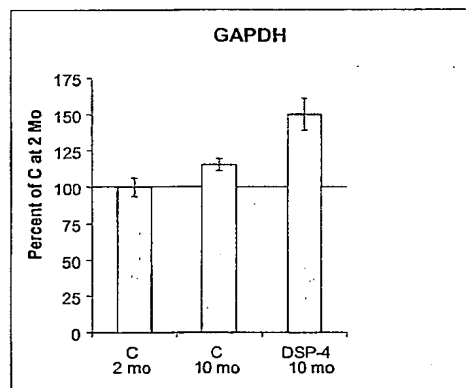


FIGURE 13C

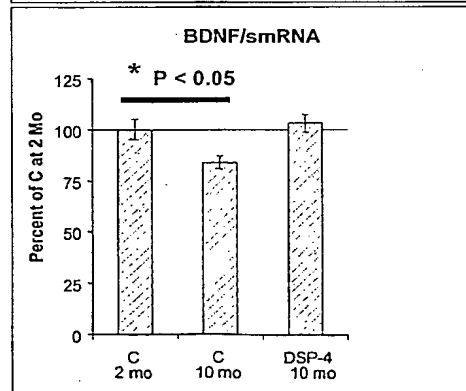


FIGURE 13D

6/7

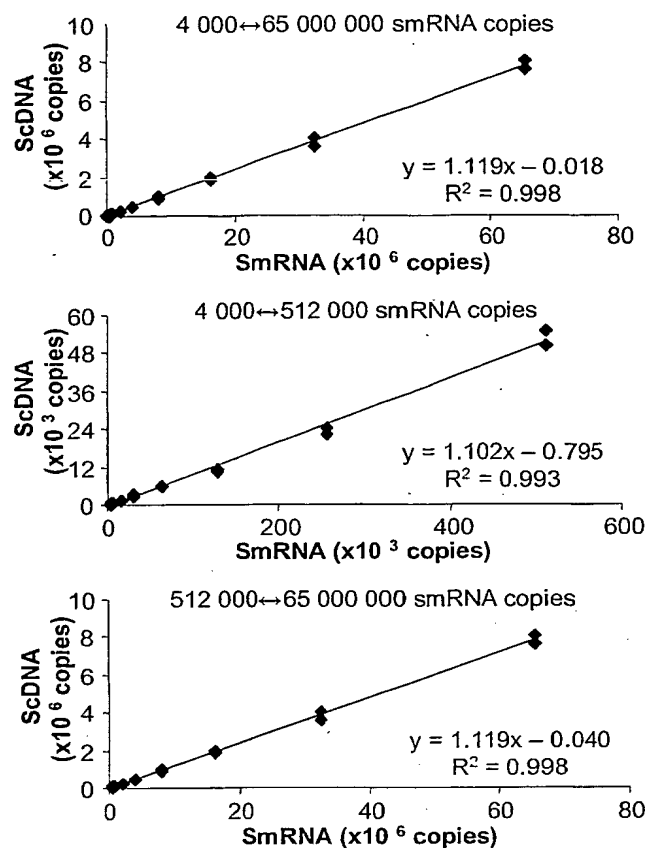


FIGURE 14

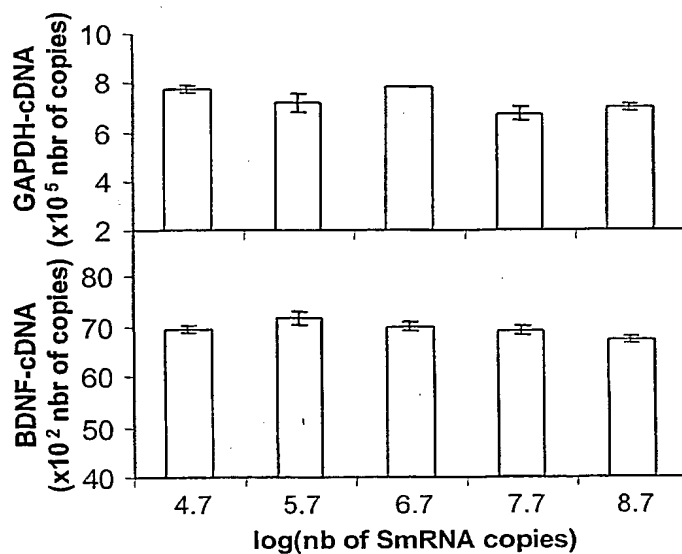


FIGURE 15

7/7

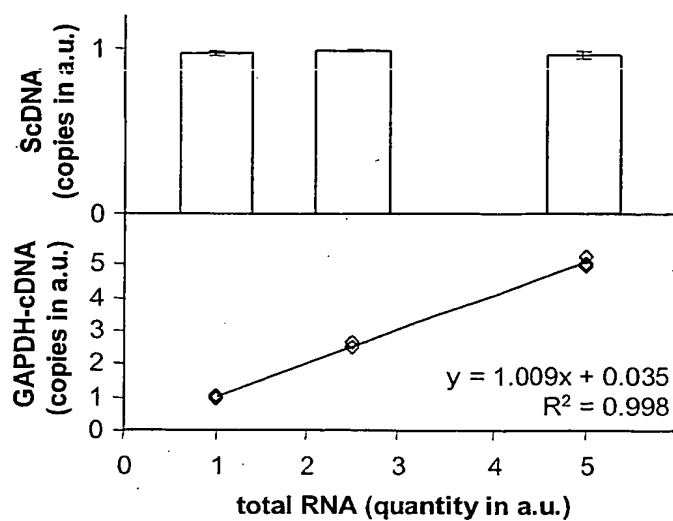


FIGURE 16

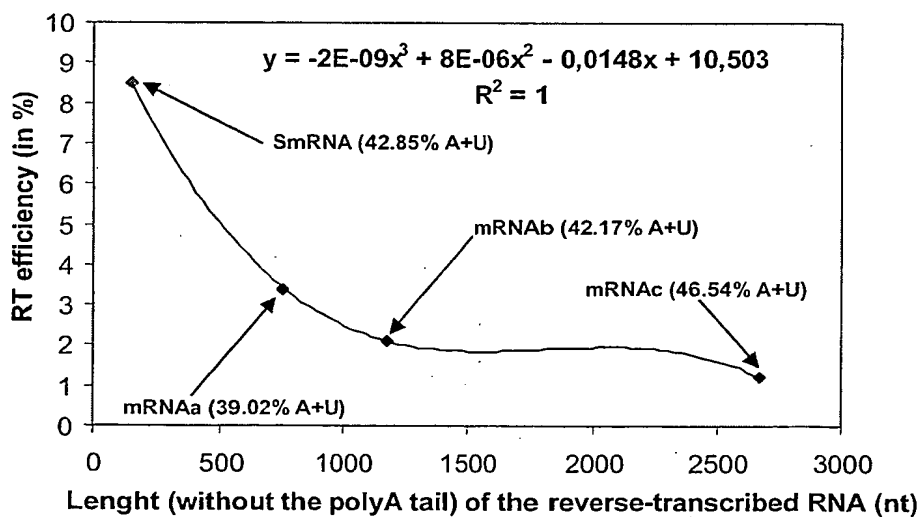


FIGURE 17

SEQUENCE LISTING

JC09 Rec'd PCT/PTO 17 OCT 2009

<110> CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE - CNRS

<120> Method of calibration of reverse transcription using a synthetic messenger RNA (smRNA)

<130> D21194

<150> EP 03/290 958

<151> 2003-04-17

<160> 18

<170> PatentIn version 3.2

<210> 1

<211> 161

<212> RNA

<213> Artificial

<220>

<223> Synthetic poly A mRNA #1

<400> 1

ggcggaauug	ggcccgacgu	cgggacaaga	agguggaaga	cgucaugcuc	ccggccgcca	60
uggcgccgc	gggaauucga	uuucuucgac	ucacugcaga	cuacugaugg	aaugacguag	120
uacgaauacu	cgacuggucu	caacaugaaa	aaaaaaaaaa	a		161

<210> 2

<211> 161

<212> RNA

<213> Artificial

<220>

<223> Synthetic poly A mRNA #2

<400> 2

uaauacgacu	cacuauaggg	cgggacaaga	agguggaaga	cgucaugcuc	ccggccgcca	60
uggcgccgc	gggaauucga	uuucuucgac	ucacugcaga	cuacugaugg	aaugacguag	120
uacgaauacu	cgacuggucu	caacaugaaa	aaaaaaaaaa	a		161

<210> 3

<211> 161

<212> DNA

<213> Artificial

<220>

<223> Synthetic cDNA #1

<400> 3

ggcggaattg	ggcccgacgt	cgggacaaga	aggtggaaga	cgatcatgctc	ccggccgcca	60
tggcgccgc	gggaattcga	tttcttcgac	tcactgcaga	ctactgatgg	aatgacgtag	120
tacgaatact	cgactggtct	caacatgaaa	aaaaaaaaaa	a		161

<210> 4

<211> 161

<212> DNA
<213> Artificial

<220>
<223> Synthetic cDNA #2

<400> 4
taatacgact cactataggg cgggacaaga aggtggaaga cgtcatgctc ccggccgcca 60
tggcgccgc gggaattcga tttcttcgac tcactgcaga ctactgatgg aatgacgtag 120
tacgaatact cgactggtct caacatgaaa aaaaaaaaaa a 161

<210> 5
<211> 19
<212> DNA
<213> Artificial

<220>
<223> Primer III forward

<400> 5
cgggacaaga aggtggaag 19

<210> 6
<211> 22
<212> DNA
<213> Artificial

<220>
<223> Primer III reverse

<400> 6
agtctgcagt gagtcgaaga aa 22

<210> 7
<211> 182
<212> DNA
<213> Artificial

<220>
<223> Sequence of the DNA probe "DNAE"

<400> 7
taatacgact cactataggg cgggacaaga aggtggaaga cgtcatgctc ccggccgcca 60
tggcgccgc gggaattcga tttcttcgac tcactgcaga ctactgatgg aatgacgtag 120
tacgaatact cgactggtct caacatgaaa aaaaaaaaaa acgcattcaa cctgtctgac 180
ta 182

<210> 8
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Sequence of the T7 promoter

<400> 8
taatacgact cactataggg 20

<210> 9
<211> 27
<212> DNA
<213> Artificial

<220>
<223> 27 pb insert

<400> 9
cgggacaaga aggtggaaga cgtcatg

27

<210> 10
<211> 34
<212> DNA
<213> Artificial

<220>
<223> 34 bp from pGEM®-T Easy sequence

<400> 10
ctcccgccg ccatggcggc cgcgggaatt cgat

34

<210> 11
<211> 101
<212> DNA
<213> Artificial

<220>
<223> 101 bp insert

<400> 11
ttcttcgact cactgcagac tactgatgga atgacgtagt acgaatactc gactggtctc 60
aacatgaaaa aaaaaaaaaa cgcattcaac ctgtctgact a 101

<210> 12
<211> 39
<212> DNA
<213> Artificial

<220>
<223> Forward primer A containing the T7 promoter

<400> 12
taatacgact cactataggg cgggacaaga aggtggaag

39

<210> 13
<211> 21
<212> DNA
<213> Artificial

<220>
<223> Reverse primer A

<400> 13
tagtcagaca ggttgaatgc g

21

<210> 14
<211> 82
<212> DNA
<213> Artificial

<220>
<223> Amplified fragment from both synthetic cDNA #1 and cDNA #2 with
primer pair III

<400> 14
cgggacaaga aggtggaaga cgtcatgctc ccggccgccca tggcggccgc gggaattcga 60
tttcttcgac tcactgcaga ct 82

<210> 15
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Pair I: primer forward

<400> 15
aattgggccc gacgtcgcat 20

<210> 16
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Pair I: primer reverse

<400> 16
catgttgaga ccagtcgagt 20

<210> 17
<211> 19
<212> DNA
<213> Artificial

<220>
<223> Pair II: primer forward

<400> 17
cgggacaaga aggtggaag 19

<210> 18
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Pair II: primer reverse

<400> 18

tcatgttgag accagtcgag

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB2004/001462

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HABERHAUSEN ET AL: "Comparative study of different standardization concepts in quantitative competitive reverse transcription PCR-assays" JOURNAL OF CLINICAL MICROBIOLOGY, WASHINGTON, DC, US, vol. 36, no. 3, March 1998 (1998-03), pages 628-633, XP002132721 ISSN: 0095-1137 abstract page 630, column 2, paragraph 2 page 631, column 1, paragraph 2 - page 633, column 1, paragraph 1; figures 3,4 ----- -/--	1-3,5-8, 11-32

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

15 July 2004

Date of mailing of the international search report

05/08/2004

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Bradbrook, D

INTERNATIONAL SEARCH REPORT

In International Application No
PCT/IB2004/001462

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91/02817 A (CETUS CORP) 7 March 1991 (1991-03-07) page 11, line 23 - page 14, line 18; figure 1; examples 1-4 page 20, lines 6-24 -----	1-3,5-8, 11-32
X	ZAMORANO P L ET AL: "QUANTITATIVE RT-PCR FOR NEUROENDOCRINE STUDIES A MINIREVIEW" NEUROENDOCRINOLOGY, S.KARGER, BASEL, CH, vol. 63, 1996, pages 397-407, XP002941018 ISSN: 0028-3835 page 400, column 1, paragraph 2 - page 406, column 1, paragraph 1; figures 1,2 -----	1-3,5-8, 11-32
X	JENSEN LISELOTTE E ET AL: "Competitive reverse transcription polymerase chain reaction for quantifying pre-mRNA and mRNA of major acute phase proteins." JOURNAL OF IMMUNOLOGICAL METHODS, vol. 215, no. 1-2, 1 June 1998 (1998-06-01), pages 45-58, XP004146492 ISSN: 0022-1759 abstract; figures 2,5 page 54, column 2, paragraph 1 - page 55, column 2, paragraph 1 -----	1-3,5-8, 11-32
X	SHEFLIN LOWELL G ET AL: "Thyroxine increases the levels of epidermal growth factor messenger ribonucleic acid (EGF mRNA) in the thyroid in vivo, as revealed by quantitative reverse transcription polymerase chain reaction with an internal control EGF mRNA." ENDOCRINOLOGY, vol. 132, no. 6, 1993, pages 2319-2324, XP002253790 ISSN: 0013-7227 abstract; figure 1 page 2319, column 1, paragraph 1 - column 2, paragraph 1 -----	1-3,5-8, 11-32
X	CLELAND A ET AL: "USE OF BOVINE VIRAL DIARRHOEA VIRUS AS AN INTERNAL CONTROL FOR AMPLIFICATION OF HEPATITIS C VIRUS" VOX SANGUINIS, S. KARGER AG, BASEL, CH, vol. 76, no. 3, 1999, pages 170-174, XP000956256 ISSN: 0042-9007 page 171, column 2, paragraphs 2,3 page 172, column 2, paragraphs 1,2 ----- -/--	1-32

INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/IB2004/001462

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 277 560 B1 (LU WEI ET AL) 21 August 2001 (2001-08-21) column 4, lines 47-55; claim 5; examples 8-10	1-32
X	----- GIBSON U E M ET AL: "A NOVEL METHOD FOR REAL TIME QUANTITATIVE RT-PCR" GENOME RESEARCH, COLD SPRING HARBOR LABORATORY PRESS, US, vol. 6, no. 10, 1 October 1996 (1996-10-01), pages 995-1001, XP000642796 ISSN: 1088-9051 cited in the application the whole document	1-3,5-8, 11-32
X	----- WO 01/16367 A (PERKIN ELMER CORP) 8 March 2001 (2001-03-08) page 13, line 1 - page 17, line 6 page 24, lines 21,22; example 2	1-32
X	----- EP 1 236 804 A (HOFFMANN LA ROCHE ; ROCHE DIAGNOSTICS GMBH (DE)) 4 September 2002 (2002-09-04) page 3, lines 11-42 page 4, lines 14-47	1-32
X	----- US 6 312 929 B1 (MCMILLAN WILLIAM A) 6 November 2001 (2001-11-06) the whole document	1-32
A	----- SIEBERT P D ET AL: "COMPETITIVE PCR" NATURE, MACMILLAN JOURNALS LTD. LONDON, GB, vol. 359, 8 October 1992 (1992-10-08), pages 557-558, XP002071876 ISSN: 0028-0836 the whole document	1-32
A	----- REISCHL U ET AL: "QUANTITATIVE PCR A SURVEY OF THE PRESENT TECHNOLOGY" MOLECULAR BIOTECHNOLOGY, TOTOWA, NJ, US, vol. 3, 1995, pages 55-71, XP000600241 ISSN: 1073-6085 the whole document	1-32

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/IB2004/001462

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9102817	A	07-03-1991	US 5219727 A	15-06-1993
			AT 178656 T	15-04-1999
			AU 653920 B2	20-10-1994
			AU 6352390 A	03-04-1991
			CA 2064906 A1	22-02-1991
			DE 69033044 D1	12-05-1999
			DE 69033044 T2	25-11-1999
			DK 497784 T3	18-10-1999
			EP 0497784 A1	12-08-1992
			ES 2132069 T3	16-08-1999
			JP 3331178 B2	07-10-2002
			JP 11123095 A	11-05-1999
			JP 2878453 B2	05-04-1999
			JP 5504886 T	29-07-1993
			US 5476774 A	19-12-1995
			WO 9102817 A1	07-03-1991
US 6277560	B1	21-08-2001	FR 2740781 A1	09-05-1997
			AU 7500496 A	29-05-1997
			BR 9611334 A	06-04-1999
			CA 2236842 A1	15-05-1997
			CZ 9801205 A3	12-08-1998
			EA 613 B1	29-12-1999
			EP 0862654 A1	09-09-1998
			WO 9717465 A1	15-05-1997
			FR 2740782 A1	09-05-1997
			JP 2000500007 T	11-01-2000
			NO 982039 A	05-05-1998
WO 0116367	A	08-03-2001	US 5952202 A	14-09-1999
			AU 3372799 A	26-03-2001
			EP 1104487 A1	06-06-2001
			WO 0116367 A1	08-03-2001
EP 1236804	A	04-09-2002	EP 1236804 A1	04-09-2002
			CA 2371672 A1	02-09-2002
			CN 1386865 A	25-12-2002
			EP 1236805 A1	04-09-2002
			JP 2002335981 A	26-11-2002
			US 2003054372 A1	20-03-2003
			US 2003165982 A1	04-09-2003
US 6312929	B1	06-11-2001	EP 1352091 A2	15-10-2003
			WO 02052030 A2	04-07-2002
			US 2002119464 A1	29-08-2002